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Investigation of Bax-VDAC Interactions and Their Role in Apoptosis

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INVESTIGATION OF BAX-VDAC INTERACTIONS AND THEIR ROLE IN APOPTOSIS

by
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ABSTRACT

Apoptosis is the process of programmed cell death. Increasing knowledge of this biological process can lead to understanding of neurodegenerative diseases and cancer alike. In mammals, this process occurs through mechanisms that are localized to the mitochondria. The release of cytochrome c is from the mitochondrial outer membrane (MOM) is regulated by pro and anti-apoptotic Bcl-2 family proteins. If the latter are successful, then cytochrome c's release into the cytosol activates a caspase cascade, resulting in apoptosis of the cell. Pro-apoptotic Bax is present in complexes that contain voltage dependent anion channels (VDACs). We hypothesized that in healthy cells, VDACs act as a restraint by creating a complex in the MOM that interferes with Bax's ability to form pores with Bak.

Drosophila Melanogaster was used as the genetic platform to create and induce mutations in the *porin* gene. The UAS/GAL4 system is used to target gene expression within any tissue of the *Drosophila* organism. We used transgenic flies using this system to enhance the expression of Bax in the eye or wing tissues of the fly. When Bax is expressed it induces apoptosis. We also used the CRISPR/Cas9 system to introduce point mutations within the BH3 domain of the *Drosophila* form of VDAC (Porin) to investigate the Bax-Porin relationship.

We then used polymerase chain reaction (PCR) and gel electrophoresis to determine the presence of the mutation.

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LIST OF ABBREVIATIONS

APAF1	apoptotic protease activating factor
Bcl-2	B-cell lymphoma (Bcl-2)
BH	Bcl-2 homology
Cas9	Caspase-9
CRISPR	clustered regularly interspaced short palindromic repeats
gRNA	guide RNA
HDR	homology direct repair
MOM	mitochondrial outer membrane
NHEJ	nonhomologous end joining
PCR	polymerase chain reaction
ssODN	single-stranded donor oligonucleotide
TAE	Tris-Acetate-EDTA
UAS	Upstream Activation Sequence
VDAC	voltage dependent anion channel

Introduction

Apoptotic Outline

Apoptosis in mammals is programmed cell death. Apoptosis can be used as a regulation mechanism to only allow developmentally appropriate and undamaged cells to live and proliferate in an effort to maintain homeostasis. Apoptosis is essential to the maintenance and development of an organism. Unneeded and damaged cells are disposed of; human adults have around 1 to 2 billion cells undergo apoptosis per hour. (Alberts, 2002).

The intrinsic pathway of apoptosis is initiated by intracellular signals and regulated by pro and anti-apoptotic B-cell lymphoma (Bcl-2) family proteins controlling the release of mitochondrial intermembrane proteins. Bcl-2 family proteins can have up to 4 conserved Bcl-2 homology (BH) domains. These proteins consist of 9 α-helical strands with similar domains when inactive (Westphal, 2013).

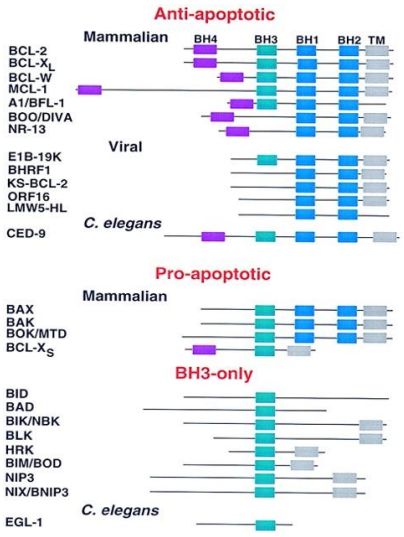

Figure 1: Homology Regions of B-cell Lymphoma 2 Family Members. Highlighted in differing colors are the conserved BH 1-4 domains of anti and pro apoptotic Bcl-2 members.

Illustration from Gross, 1999

Anti-apoptotic Bcl-2 genes are considered oncogenes due to their increased expression contributing to the characteristic longevity of cancerous cells. (Alberts, 2002). Anti-apoptotic Bcl-2 family proteins reside within the cytosolic space as well and inhibit apoptosis by binding pro-apoptotic proteins in ways that either prevent them from undergoing oligomerization on the surface of the mitochondrial outer membrane (MOM). (Alberts, 2002).

Pro-apoptotic membrane proteins Bak and Bax are BH123 proteins, meaning that they have three of the four conserved BH domains. In mammals, these proteins form the oligomers through which the cytochrome c exits the MOM. A distinct difference in these family members is that Bak is constitutively present in the MOM while Bax is present in the cytosol and is moved to the MOM when signaled. The pro-survival protein Bcl-2 can inhibit the proapoptotic proteins Bax/Bak. It is not fully known whether these proteins overcome the antagonistic effects of Bcl-2 to induce apoptosis or their activation alone induces apoptosis (Figure 2, b). (Gaumer, 2000).

However, there is another subfamily of pro-apoptotic proteins the BH3-only proteins. BH3-only proteins promote apoptosis by binding and neutralizing the anti-apoptotic Bcl-2 proteins, and by binding to and activating Bax and Bak. The Bax and Bak proteins then can form an oligomer on the MOM. The oligomerization of Bax and Bak occurs by the exposed BH3 domain of one protein interacting with a groove within the

second molecule. These BH3 domain interactions lead to the formation of an oligomer that allows the membrane to be permeabilized (Alberts, 2002; Westphal 2013).

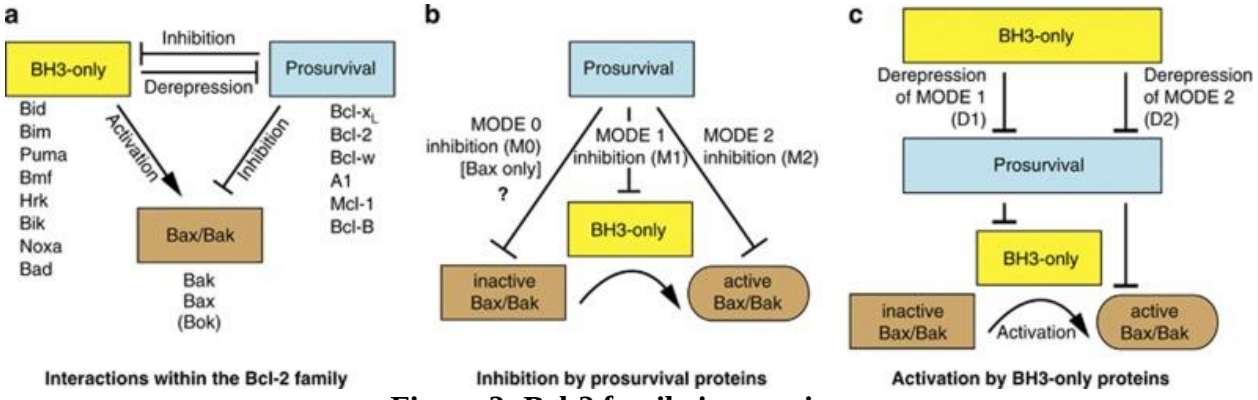

Figure 2: Bcl-2 family interactions.

(a) Proapoptotic Bax/Bak and BH3 only proteins are shown. Anti-apoptotic Bcl-2 proteins inhibit the activity of proapoptotic Bcl-2 family members. (b) Apoptosis can be inhibited by Bax being bound and brought to the cytosol, BH3 only proteins can be inhibited from activating Bax/Bak, or Bax and Bak can be bound to prevent their oligomerization. (c) BH3 proteins activate Bax/Bak by directly binding them or competing with anti-apoptotic proteins.

Illustration from Wesphal, 2013

The mechanism by which Bax/Bak form a pore on the MOM is unclear, but there is evidence that they interact with voltage dependent anion channel (VDAC) 1 and 2 to accomplish this. VDAC is an abundant protein on the surface of the mitochondria that serves as not only a pore for metabolites but also interacts with pro and anti-apoptotic proteins. VDAC2 is thought to enhance recruitment of Bak to the MOM and also keep Bak in its inactive state. Many healthy cells have MOM-inserted Bak that is associated with VDAC2. Pro-apoptotic Bax is present in complexes that contain VDAC 1 and 2 on the MOM in healthy cells. These Bax-VDAC complexes are what we hope to investigate further with our research. (Camara 2017; Westphal, 2013).

As a result of the Bax/Bak pore formation, cytochrome c is released into the cytosol of the cell which leads to the activation of a caspase cascade (Figure 3). The combined loss of functionality of the energy creating pathway of the cell due to the loss of the polarization of the membrane needed to power electrochemical gradient is enough to cause the cell considerable losses. The release of this material activates a caspase cascade to start cell death. The cascade is activated by Cytochrome c binding the apoptotic protease activating factor 1 (APAF1) protein which then oligomerizes into an apoptosome. Executioner caspases begin in the inactive procaspase phase but are then cleaved and therefore activated by an initiator caspase like Caspase-9 (Cas9). The cleavage of these executioner caspases induces a cascade of proteolytic cleavage, resulting in the breakdown of cellular components. (Alberts, 2002)

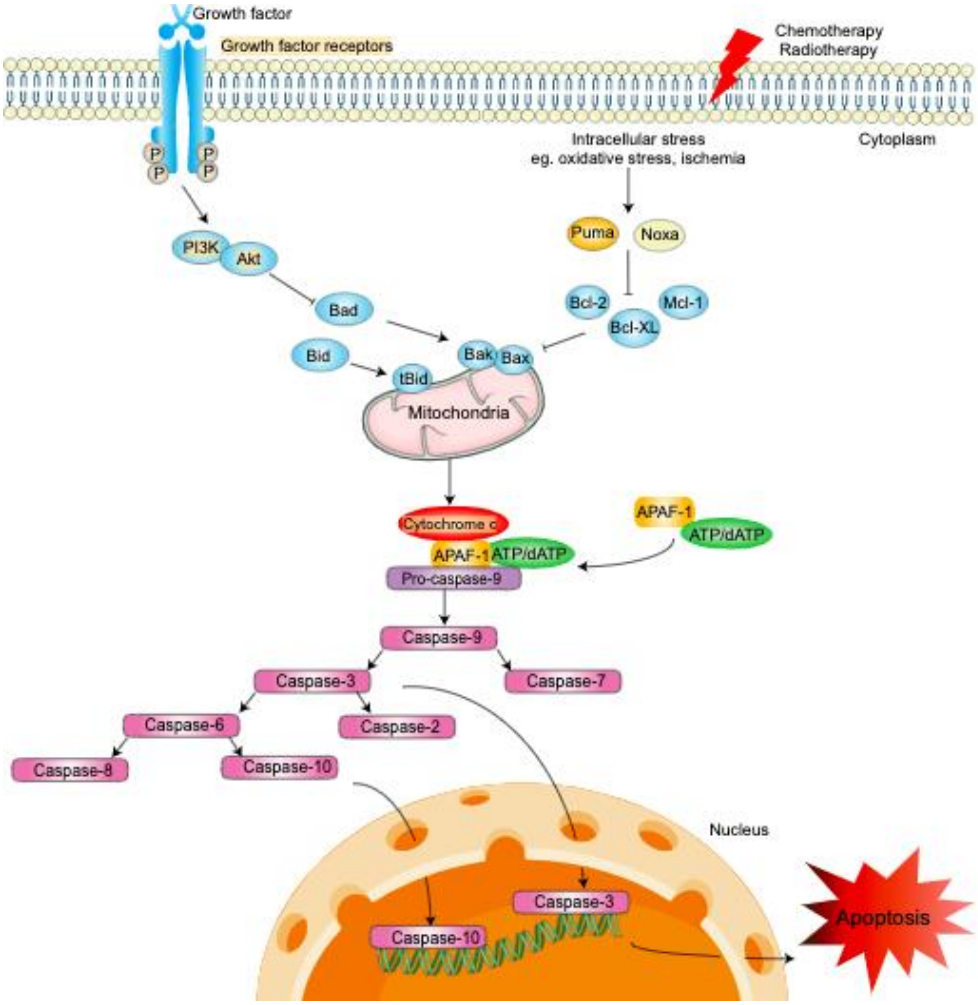

Figure 3: Apoptotic Pathway Throughout the Cell. The intrinsic apoptosis pathway is activated by stimuli such as DNA damage, ischemia, and oxidative stress. Pro-apoptotic signaling causes the release of cytochrome c into the cytoplasm. Bcl-2 proteins regulate this process at the MOM. When in the cytoplasm, cytochrome c forms an apoptosome with apoptotic protease activating factor 1 (APAF1) and the inactive form of caspase-9. Cas9 is activated and then cleaves and activates the executioner caspases-3/6/7, resulting in cell apoptosis.
Illustration from Creative Diagnostics

Investigating Bax-VDAC Relationship

We hypothesized that in healthy cells, VDAC proteins in the MOM acts as a restraint on Bax by creating a Bax-VDAC complex in the MOM that interferes with Bax's ability to oligomerize and form pores. The absence of activated Bax in Bax-

VDAC 1 complexes in healthy cells represents that Bax is being prevented from prematurely assembling complexes in the MOM. We have recently discovered that the VDAC protein appears to have a BH3-like domain on its N-terminus (Figure 4). We would like to investigate this part of the VDAC protein and to determine if the domain is an actual BH3 domain where the proteins are interacting. (Jekabsons and Jones, 2019)

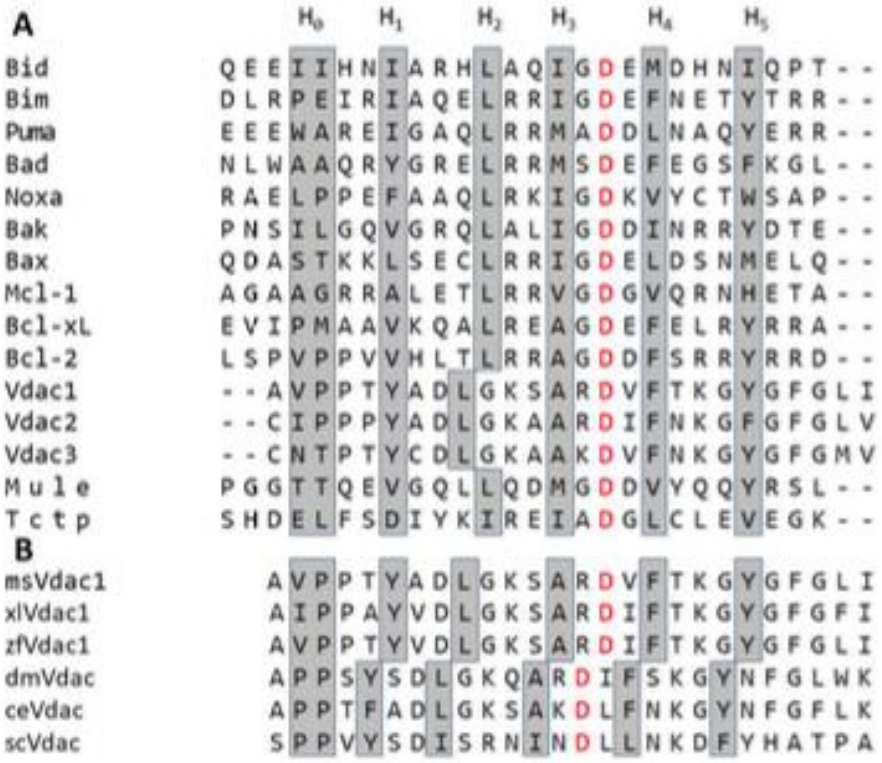

Figure 4: Conserved Regions of Bcl-2 Family Members and VDACS. Hydrophobic regions H0-5 are shown. The conserved aspartate (D) is in red. It is hypothesized that VDAC has a BH3 domain similar to Bcl-2 family proteins. Figure from Jekabsons and Jones, 2019

To investigate the relationship between Bax and VDAC, the model organism of *Drosophila* was chosen because it is well-studied with many tools available to manipulate gene expression with. In addition, *Drosophila* has Bax and VDAC homologs, and mammalian Bax can induce apoptosis when expressed in the compound eyes or in the wings of *Drosophila*. Due to the presence of eyes and wings being unessential to the

Drosophila's survival, it is possible to genetically manipulate the components of apoptosis in them and evaluate what happens phenotypically.

The UAS/GAL4 system is used to target gene expression within any tissue of the *Drosophila* organism. GAL4 is a transcription factor found in yeast that encodes a protein that will bind to an enhancer called the Upstream Activating Sequence (UAS) to activate gene expression. Under the control of tissue specific enhancers GAL4 can be expressed in Drosophila target organs such as the eyes or wings of a fly. The exogenous yeast GAL4 is used because it has no endogenous targets within *Drosophila* DNA, meaning that it does not affect gene expression without UAS and its target gene present. However if yeast enhancer UAS is present, it acts as an upstream activating sequence when its GAL4 binding sites are bound and activates expression of the target gene. This system is turned on when a fly with GAL4 expressed in particular cells is crossed with another fly containing UAS-Bax (Figure 5) for example. (Brand, 1993).

The GMR-GAL4 line is expressed in the eye and was crossed with UAS-Bax to generate offspring with GMR-GAL4; UAS-Bax. This turns on the expression of the Bax gene in the eye which creates more pro-apoptotic proteins and results in a rough appearance of the eye (Figure 5, B). It is unknown why the occurrence of apoptosis is increased with higher expression of Bax. The Bax protein is lethal and requires activation by interacting with other proteins in the MOM.

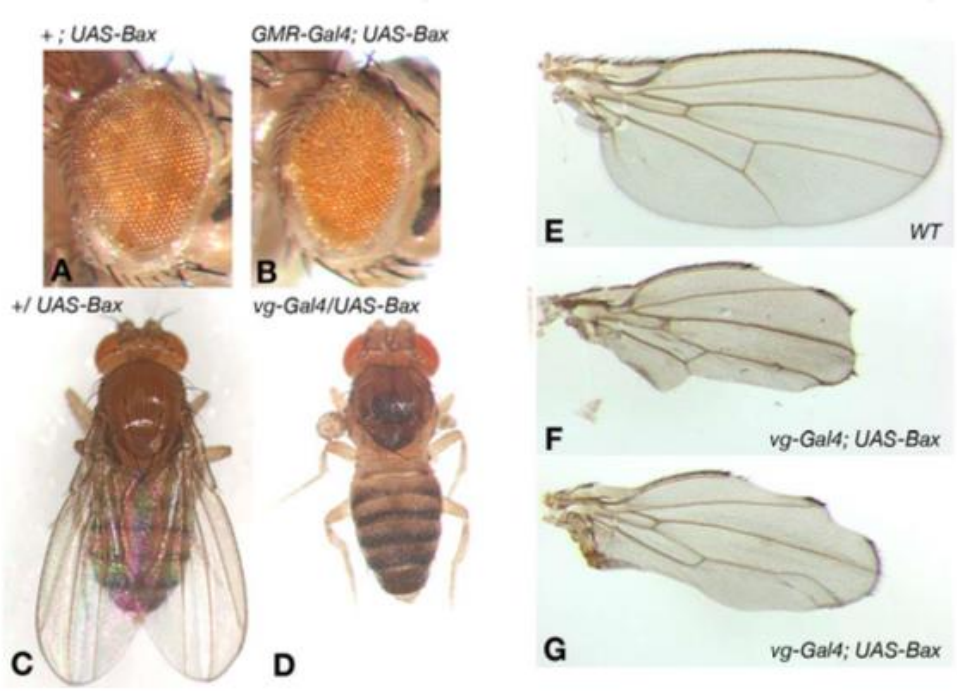

Figure 5: Phenotypes of Transgenic Flies

(A) A picture of a fly's eye with UAS-Bax, but no GMR-GAL4 genomic enhancer activating its expression. (B) A picture of a transgenic fly's eye with a rough appearance due to tissue-specific increased cell death. This is activated by increased Bax expression due to the UAS/GAL4 systems presence. (C) A picture of a fly's wings with UAS-Bax, but no tissue specific vg-GAL4 genomic enhancer activating its expression. (D) A picture of a fly's wings after increasing tissue-specific expression of the Bax gene. (E), (F), and (G) show the phenotypic variation of flies more weakly expressing the Bax gene when compared to (D) Figure from Jekabsons and Jones, 2019

To test for interactions between Porin and Bax the CRISPR/Cas9 system was used to introduce point mutations within the BH3 domain of the *Drosophila* form of VDAC (Porin). After the *porin* gene was mutated, we planned to cross UAS-Bax with GMR-GAL4 in flies with the *porin* mutation to see how this would affect the phenotypes. If Porin is required for Bax functions, then we should see a phenotypic change with mutations in the *porin* gene. The eyes of the fly could be rougher or get better depending on the function of Porin in the enhancement or suppression of the Bax protein's function.

Clustered regularly interspaced short palindromic repeats (CRISPR) allow genetic modification to be done in a precise manner. Breaking the DNA in this way allows for repair either by nonhomologous end joining (NHEJ) or homology-direct repair (HDR) that can be used to edit the genome. This process needs guide RNA (gRNA) that is complementary to the Porin gene to indicate where the break will be on the second chromosome. The Cas9 protein is directed by a gRNA to cause a double strand cleavage of DNA in a specific spot. At the cut site, specific ssDNA or dsDNA repair templates that are homologous to the end of the double strand break can be used to start HDR rather than NHEJ. Ideally, the cell will find this copy with the new allele that is being introduced and use it for a template for DNA repair. This system will be used to cut and introduce mutations within the Porin gene.

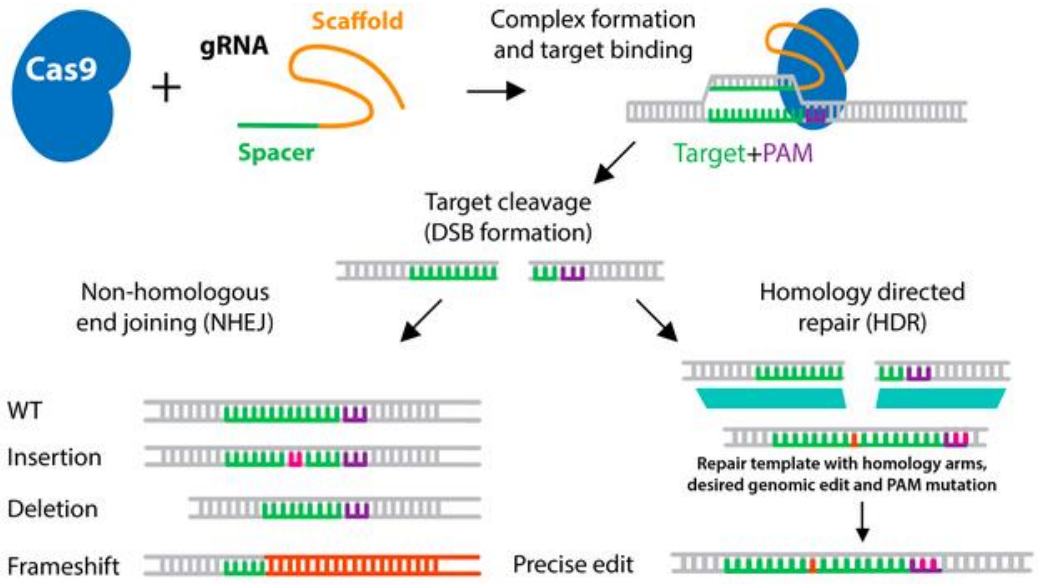

Figure 6: Required Components for CRISPR and NHEJ vs. HDR. The Cas9 protein as well as a gRNA are the basic components for the CRISPR/Cas9 system. This system induces a DSB that can be repaired in one of two ways: NHEJ or HDR which are pictured here. It was our goal to create a system that would use the HDR method of repair to incorporate Porin mutations into the genome.
Illustration from addgene

Included in the plasmid that we used to create the gRNAs for the CRISPR/Cas9 system is a strong U6:3 promoter for RNA polymerase III which drives ubiquitous expression of RNA genes without incorporating a poly-A tail and a cap which we did not want added. The plasmid will be cut with Bbs1 and the DNA with the target sequence will be inserted within the Bbs1 spacer region. The plasmid containing must then be injected along with the oligonucleotide necessary for HDR into fly embryos that express Cas9; the plasmid is transcribed to generate multiple gRNAs The single-stranded donor oligonucleotide (ssODN) injected has all of the mutations within it and is used to repair the DSB caused by Cas9. The Cas9 protein is only present in the germ line cells, because we do not want cutting induced in adult flies, we just want the system to alter the DNA of cells becoming eggs and sperm. (Gratz 2015).

Much of the barrel-shaped Porin protein is unaltered, but the BH3-like domain of the protein that we hypothesize to interact with Bax is being mutated. One of the point mutations we are trying to introduce into the Porin gene using CRISPR changes a conserved, negatively charged aspartic acid (D) to an uncharged glycine. The second is a point mutation of the BH3 domain's hydrophobic amino acid side chain leucine (L) to a glycine (G). These mutations would change some of the protein's highly conserved amino acids within its domain and possibly cause the protein to be unable to form the Bax-VDAC complex. (Jekabsons and Jones. 2019).

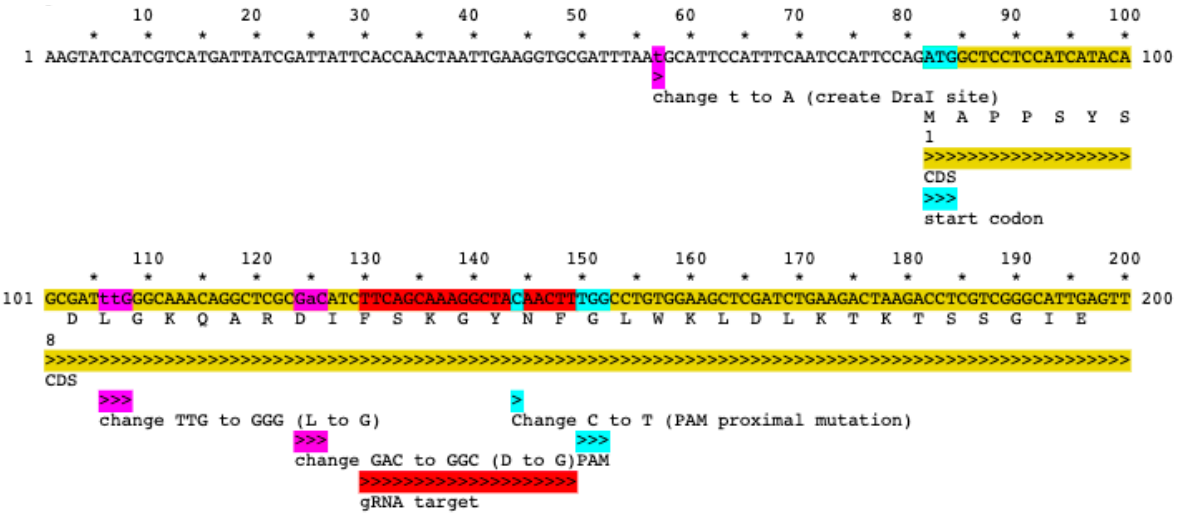

Figure 7: Region of Porin BH3 Domain Mutations. The red region of DNA is our target gene and underneath is the gRNA target. In the magenta regions, a hydrophobic Leucine amino acid will have its codon changed from TTG to GGG so that it will now have a Glycine side chain. The other magenta region is changing a charged Aspartic Acid codon from GAC to GGC so that it will have a Glycine side chain as well. The PAM region is highlighted in blue. The corresponding point mutation before it was placed there so that after repair, Cas9 won't come back and cut there again because the gRNA will not line up.
Illustration by Dr. Brad Jones

To see if the system we have introduced has successfully incorporated the nucleotide changes from the injected oligonucleotide into the genomic DNA at the *porin* locus, we must screen through fly lines derived from the injected fly embryos. To detect flies that have the incorpaorated mutations, the ssODN would also introduce a unique restriction site for the enzyme DraI (Figure 7). This restriction site will allow DraI to cut the DNA fragment generated by PCR into two smaller fragments resulting in two regions of DNA at lower weights showing up on an agarose gel. One section will be 362 base pairs (bp) while the other will be 763 bps.

Materials and Methods

Testing Procedures

Fly Preparations

To ensure that the target genotype had been obtained, F1 flies derived from the injected embryos were tested. 1 to 10 *Drosophila* were selected from each line and put into an Eppendorf tube, then placed into an -80 °C freezer for 5 minutes. Once frozen, 100 µl of Buffer A (100mM TRIS, 100mM EDTA, 100mM NaCl , 0.5% SDS) was pipetted into the tube and the flies were homogenized using a mini-pestle. This step is essential to break apart and dissolve the cell components that encase the DNA, making it available for use. After homogenization, an additional 100 µl of Buffer A was pipetted into the tube and it was put into a 65 °C incubator for 20 minutes. This allowed the protein contents of the fly to further breakdown. 40 µl of 1.5M K acetate 4.5M LiCl was added to the solution as it rested on ice for 20 minutes. The 1.5M K acetate 4.5M LiCl solution was used to make the proteins insoluble, detergents, and other smaller cellular components insoluble to water. The mixture was then centrifuged for 15 minutes at 13,000 RPM. The insoluble cellular components were pelleted at the bottom of the tube while the DNA remained soluble. The DNA-containing supernatant was obtained by pipetting 100 µl from the top of the tube and placing it into a new one. The precipitation process was done by adding 200 µl of EtOH to the supernatant and then spinning it again in the centrifuge at 13,000 RPM for 15 minutes. The negatively charged backbone of the

DNA is ionically bound to the positive ions, causing its precipitation from the aqueous solution. The supernatant was discarded and the desired outcome of this process is a pellet of DNA at the bottom of the tube. The DNA pellet was rinsed to remove unbound salt ions by using 200 μl of 70% EtOH. Once the remaining ethanol had evaporated and the pellet was dry, the DNA was resuspended in 50 μl TE solution.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify the 1125 bp DNA sequence that was edited to determine if the CRISPR/Cas9 system had successfully introduced point mutations into the *Drosophila* genome. A PCR mix containing 25 μl of 5' primer, 25 μl of 3' primer, 25 μl of PCR Buffer, 25 μl of 2 mM dNTPs, and 135 mL diH_2O was used. Each PCR tube had 23.5 μl of PCR mix, 1.0 μl of the template DNA diluted 1:10 in H_2O, and 0.5 μl TAQ polymerase. A specific program was used with the following temperature and time cycles:

Step #	Temperature (°C)	Time (sec)
1	94	300
2	94	30
3	62	30
4	72	120
5	Go to step 2, 34 times	--
Step #	Temperature (°C)	Time (sec)

6	72	420
7	4	--

Table 1: PCR Setup.

The plasmids were designed so that if the mutated sequence was incorporated via CRISPR/Cas9, there would be a specific enzyme cut site that is flanked by the primers added. The restriction enzyme DraI was used to cut at this specific place in the DNA. 0.5 µl of DraI enzyme (New England Biolabs) was added to each PCR tube and incubated at 37 °C. .

Gel Electrophoresis

Gel electrophoresis separates DNA based upon its number of base pairs affecting its speed; the shorter a DNA fragment is the faster it will travel. The negative DNA travels across the agarose gel towards the positively charged electrode and way from the negatively charged electrode. To create the gel, 1.5 grams of agarose per 100 ml Tris-Acetate-EDTA (TAE) were dissolved by heating, then 0.75 µl EtBr were added. The DNA ladder was prepared by pipetting 10 µl of pre-prepared 1:10 Kb ladder with loading dye into the first well of the gel. The samples were prepared by pipetting 1 µl of PCR product, 1 µl gel loading dye, and 8 µl of water into a tube and then into their respective wells within the agarose gel. 120 volts were applied to facilitate the movement of DNA.

Results

The tasks that I was doing in lab were in preparation to screen through the lines that had been injected to see if the CRISPR/Cas9 mutations had been integrated into the fly's genome. I needed to be able to consistently and reproducibly carry out fly preps and then PCR to get results. I was using multiple fly lines and doing preps of different dilutions of flies. The number of flies differed so that when we were testing later, I could reliably achieve results with multiple flies so that we could ascertain the frequency of the mutation in the F1 generation. I was able to successfully preform the protocol and obtain PCR bands on an agarose gel.

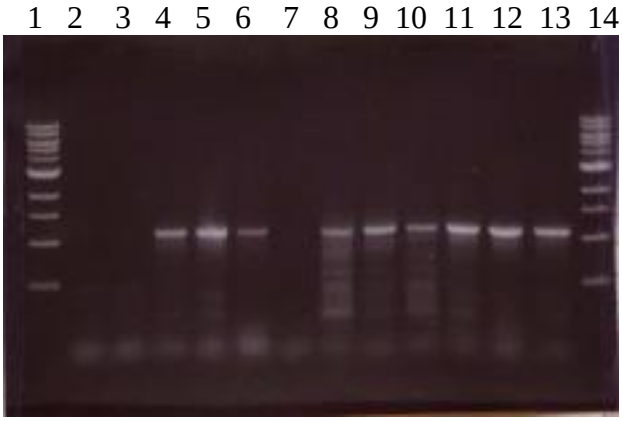

Figure 8: PCR of fly preps with multiple dilutions. Lanes 1 and 14 contain 1 Kb ladder solution. Lanes 2-6 are undiluted. Lane 2 contains *porin* gRNA #1 and lane 3 contains *porin* gRNA #2. Lane 4 contained 1 fly (nos-cas9). Lane 5 contained 5 flies (nos-cas9). Lane 6 contains 10 flies (nos-cas9). Lanes 7-13 are diluted. Lane 7 contains w^{1118} 1:10. Lane 8 contains 1:10 diluted *porin* gRNA #1 and lane 9 contains 1:10 diluted *porin* gRNA #2. Lane 10 contains 1:10 dilution with 1 fly. Lane 11 contains 1:10 dilution with 5 flies. Lane 12 contains a 1:10 dilution with 10 flies. Lane 13 contains w^{1118} diluted 1:10.

Discussion

The results I have yet to obtain were going to be screenings of the injected fly lines. The international COVID-19 crisis has made this impossible to do before the semester is over because of precautions taken by the University in suspending all physical classes and moving to an online format. Dr. Jones had injected GMR-GAL4 and UAS-Bax fly embryos with the material for CRISPR/Cas9 system. The lines were then crossed to produce progeny with the GAL4/UAS induced over-expression of Bax and CRISPR/Cas9 system mutated Porin. It would then be my responsibility to screen through the offspring to see if any of the progeny had successfully integrated the mutations into their genome. The screening would be done by obtaining DNA from each fly line and using PCR to multiply the *porin* gene and gel electrophoresis to see the resulting weights of the DNA.

The 1125 bp sequence that would be replicated with PCR is shown in figure 9. The sequence starts with the blue region representing the 5' primer and ends with the green region representing the 3' primer. The introduced mutations are in closer to the middle of the sequence. The DraI cut site is around 360 bp and highlighted in pink, showing where the DNA will be cut if the mutation is successfully incorporated.

If the CRISPR/Cas9 system had incorporated itself into the DNA, the restriction enzyme DraI would cut the DNA at the cut site. The result of this would be that two smaller bands of DNA would appear on the gel electrophoresis if a homozygous mutation

had occurred. One band would be around 360 bp while the other around 760 bp. The mutation is likely to be heterozygous, meaning that a larger and brighter band would be seen on the gel at around 1125 bp but then the two smaller bands would also be more faintly present. A goal of our experiment was to test the genetic lines to see if they had successfully integrated the CRISPR/Cas9 system.

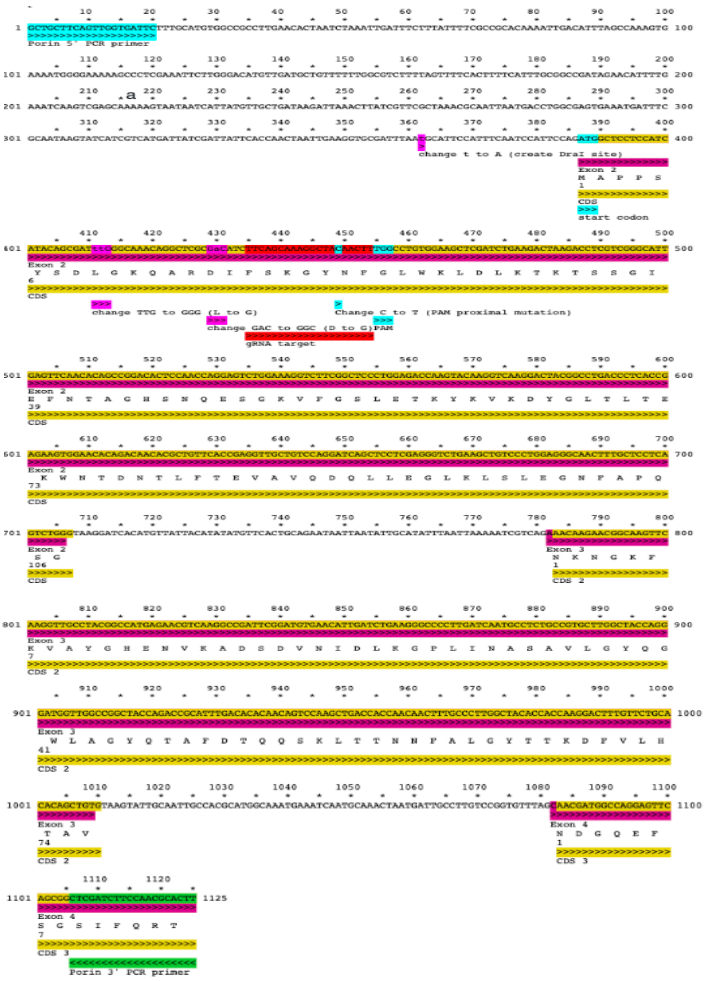

Figure 9: Region of *porin* Gene Replicated with PCR. The region of the *porin* gene that was multiplied using PCR techniques.
Illustration by Dr. Brad Jones

Our hope in screening the 20 lines that were injected is that at least one would have the incorporated mutation. Unfortunately, our research ended promptly in March

not allowing me to get past the preparation phase for this experiment. If continued, lines with mutations detected would be crossed and screened through continually with the goal of increasing the number of flies with the mutation. Eventually, we would hopefully have fly lines that were homozygous with the inserted mutation.

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